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Characteristics of Hepatitis C Virus Before and After Interferon Treatment in Patients With Ongoing Viraemia but Sustained Biochemical Response

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In hepatitis C virus (HCV) infection, persistent viraemia can occur after successful biochemical response to interferon treatment. To assess whether this unusual profile might be due to trivial amounts of remaining virus or to the emergence of less pathogenic HCV strains, pre- and posttreatment sera from 27 patients who remained with HCV-RNA, despite sustained transaminase normalisation after interferon therapy, were investigated. All but one had infection by genotype 2 ($P < 0.0001$), and levels of HCV-RNA were not decreased after therapy. Sequence comparison of the 5′ untranslated region revealed mixed viral populations and ''not compensatory'' nucleotide transitions localised at the stem level of the secondary structure of this region in samples taken before and after treatment. Neither quantitative nor qualitative viral changes, at least for the 5′ untranslated region, are responsible for interferon-induced biochemical remission in these patients typically infected by genotype 2. ***J. Med. Virol. 54:7–11, 1998.***

KEY WORDS: HCV infection; asymptomatic carriership; viral load; 5′UTR sequence analysis

INTRODUCTION

Interferon (IFN) therapy has been shown to induce a sustained normalisation of transaminases in about 20–30% of patients with chronic hepatitis C, but 10–20% of these long-term responders remain HCV-RNA positive in serum [Lau et al., 1993; Conjeevaram et al., 1995]. The significance of persistent viraemia with normal transaminases after therapy is still poorly understood. Recent data indicate that this profile is frequently associated with late relapse of liver disease [Chemello et al., 1996]. However, selection of viral strains with different pathogenicity may occur after IFN therapy [Simmonds et al., 1995], and patients with a sustained biochemical response to IFN with ongoing HCV replication represent a good model to evaluate possible viral changes associated with transaminase normalisation. Therefore, we compared the virus load and type before and after treatment in a group of patients with normal transaminase profile and persistent viraemia after IFN therapy. In some, we also analysed whether changes in the virus genome had occurred during treatment. In particular, we compared, before and after therapy, the sequence of that part of the 5′ untranslated region (5′UTR) that contains an internal ribosomal entry site (IRES) responsible for the control of virus translational process [Tsukiyama-Kohara et al., 1992; Wang et al., 1993]. HCV genotypes showing different sequence motifs in this region may have a different efficiency of protein translation [Tsukiyama-Kohara et al., 1992; Gerotto et al., 1997]. Thus, nucleotide variations could influence the expression of the viral polyprotein and, consequently, viral pathogenicity.

PATIENTS AND METHODS

Patients

The patients included in the present study were derived from a large series of 114 cases who had been treated with IFN in randomised clinical trials conducted in our institution between 1989 and 1993 and had a sustained biochemical response after therapy, with persistent transaminase normalisation main-

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tained up to 12 months after cessation of IFN [Casarin et al., 1994; Chemello et al., 1995]. Twenty-seven (24%) of the 114 patients had HCV-RNA in serum after therapy, despite the complete normality of ALT, and were therefore selected for the present study. Sixteen (14%) patients already had HCV-RNA at the end of treatment and 11 additional patients became positive during the posttreatment period. For this reason, in the 27 HCV-RNA-positive patients, comparison between pre- and posttreatment HCV-RNA levels was assessed in samples obtained before and within 6–12 months after treatment.

Viral Markers

Anti-HCV was assessed by second generation enzyme-linked immunosorbent assay (ELISA-2) (Ortho Diagnostic Systems, Raritan, NJ) and by second generation immunoblot assay (RIBA-2) (Chiron Corporation, Emeryville, CA). HBsAg and anti–human immunodeficiency virus were determined by ELISA (Abbott Laboratories, North Chicago, IL).

HCV-RNA Detection

HCV-RNA was studied in serum by nested polymerase chain reaction after reverse transcription (RT-PCR). The 5′UTR primers used for PCR and sequencing were synthesised with an Applied Biosystem model 394 DNA/RNA synthesiser, as described elsewhere [Tisminetzky et al., 1995].

Briefly, 3 μl of serum were mixed with 21 μl of 50 mM buffer Tris-HCl (pH 8. 3), 50 mM KCl 2.5 mM $MgCl_2$, 1 mM of the four dNTPs (Boehringer Mannheim, Germany), 30 U of RNase inhibitor (Boehringer Mannheim), and 50 pM of the antisense external primer AS1 (5′-GTGCACGGTCTACGAGACCT-3′) and were heat treated for 90 sec at 94°C. After quick chilling, 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Milano, Italy) were added and incubated for 1 hr at 37°C. All 25 μl of cDNA were used for the first step of amplification in a reaction volume of 100 μl containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 2.5 U of Taq DNA Polymerase (Boehringer Mannheim), 50 pM of both external primers AS1 and S1 (5′-GCCATGGCGTTAGTATGAGT-3′) and were overloaded with 100 μl of mineral oil (Sigma Chemicals, Milano, Italy). After one cycle of amplification of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, 25 additional cycles at 94°C for 45 sec, at 50°C for 45 sec, and at 72°C for 45 sec were run. For the second round of amplification, an aliquot of 3 μl of the first step of the PCR reaction was amplified in the same conditions described in a heminested PCR using an antisense internal primer AS2 (5′-GGCACTCGCAAGCACCCTAT-3′) and the S1 primer. PCR products were visualised by ethidium bromide staining after electrophoresis in a 2% agarose gel. Water and serum from normal healthy subjects were used as negative controls. All reagents were treated with ultraviolet light, and filter tips were used to minimise contamination.

Direct Sequencing

For direct sequencing, a recently available method was used (Amersham, UK) in which PCR products were pretreated with esonuclease I and shrimp alkaline phosphatase at 37°C for 15 min, followed by incubation at 80°C for 15 min to inactivate both enzymes. This approach allowed us to avoid further purification steps of the amplified product and, consequently, to avoid material loss that can occur during the DNA purification process. Direct sequencing was then performed by adding either the AS2 or S1 specific primer to 5 μl of treated PCR product (about 0.5 pmol of DNA). The Sequenase PCR Product Sequencing Kit (Amersham) was used, and ^{35}S-dATP was included in the labelling reaction. After acrylamide gel electrophoresis and overnight exposure of the autoradiographic film, nucleotides from −60 to −210 map positions of the 5′UTR [Choo et al., 1991] were clearly read in each case. All samples were amplified and sequenced 2–3 times, using both sense and antisense primers to confirm the results.

The program MFOLD was used to assess the secondary structure of the 5′UTR sequence obtained and to calculate its free energy (DG) in selected cases.

HCV Genotyping

HCV genotype was evaluated by using the previously described dot-blot assay [Pontisso et al., 1995b] in which type-specific probes, derived from the highly variable region of 5′UTR, were labelled with fluorescein-dUTP and hybridised with RT-PCR amplified products spotted on nylon filters. Type-specific probes were as follows: type 1 (5′-CGCTCAATGCCTGGAGAT-3′), type 2 (2a/c+2b mixed probes; 2a/c:5′-CACTCTATGCCCGGCCAT-3′; 2b:5′CACTCTATGTCCGGTCAT-3′), and type 3 (5′CGCTCAATACCCAGAAAT-3′). To reveal the labelled hybrids, the enhanced chemioluminescent method (Amersham), followed by autoradiography, was used.

HCV-RNA Quantitation

Viral load was assessed by a recently developed PCR system that includes a quantitation standard that is coamplified with the target HCV-RNA (Amplicor HCV Monitor, Roche Molecular Systems, Inc., Branchburg, NJ) [McGuiness et al., 1996]. Briefly, RNA was extracted from serum previously mixed with the quantitation standard (QS) and precipitated with isopropanol. Reverse transcription and PCR amplification of the 5′UTR was carried out in a single tube by using rTth polymerase in a 9,600 thermal cycler (Perkin-Elmer, Norwalk, CT). Amplified products were then denatured and transferred to the detection plate. Serial fivefold dilutions were carried out, and parallel hybridisation with 5′UTR-specific and QS-specific probes coated on the microwell plate was performed, followed by enzymatic detection and optical density measurement at 450 nm. Computer-assisted analysis of the results al-

TABLE I. HCV-RNA Levels Observed in 27 Sustained Biochemical Responder Patients With Ongoing HCV Replication*

	Before treatment (copy n/ml)	After treatment (copy n/ml)
Mean ± SD	38,637 ± 96,459	97,880 ± 213,195
Median	7,642	30,716
Range	2,000–404,472	2,000–894,413

$P = 0.0045$, Wilcoxon matched-pairs test.
*Before vs. after treatment.

lowed us to identify from 10^3 to 10^5 copies of HCV-RNA per millilitre in a linear range.

Paired sera from the same patient, obtained before and from 6 to 12 months after treatment were analysed in the same run of the assay.

Statistical Analysis

To evaluate the results, the Wilcoxon matched-pairs test and the Fisher's exact test were used.

RESULTS

HCV Genotype

Among the 114 patients with biochemical sustained response to IFN, genotype distribution, determined before therapy, was as follows: 16% were infected by type 1, 48% by type 2, 23% by type 3, and 25% remained unclassified, being HCV-RNA negative. When HCV genotype was evaluated in the 27 patients with HCV-RNA positivity after treatment, all but one were infected by HCV type 2. Epidemiological factors in this group of viraemic patients were similar to those of the remaining patients, whereas the observed different genotype distribution vs. that of the whole group of biochemical sustained responders was statistically significant ($P < 0.0001$).

In all the 27 patients, viral type was the same before and after treatment.

Quantitative Determination of Viral Load

Despite a wide range of HCV-RNA levels in individual patients, the amount of HCV-RNA in serum was unchanged or increased after treatment in the majority of the patients (Table I).

Comparison of HCV-RNA Sequences

Paired serum samples obtained before and after treatment were analysed by sequencing of the 5′UTR in 14 patients. Figure 1 shows the results of sequence alignment. Simultaneous presence of two nucleotides at the same position, suggesting mixed viral population, was repeatedly observed (Fig. 2). Presence of a mixed viral population was detected either before or after treatment with normal transaminase levels. Viraemic values were usually higher in these samples than in samples with a more homogeneous viral population (median copy n/ml: 47,811 vs. 17,232), and in individual patients the occurrence of dual population

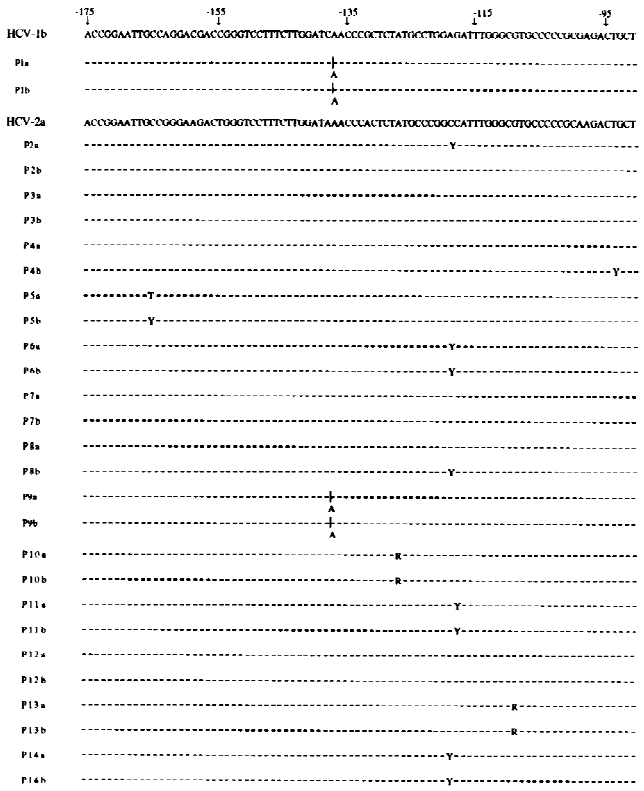

Fig. 1. Alignment and comparison of the 5′UTR sequence in paired serum samples obtained before **(a)** and after **(b)** treatment in 14 patients (P1–P14) with sustained biochemical response in relation to the corresponding HCV-1b [Choo et al., 1991] and HCV-2a [Enotomo et al., 1990] prototypes. Nucleotide numbering corresponds to that described for the prototype HCV-1 sequence [Choo et al., 1991]. In patients P1 and P9, A insertion at -138 has been indicated. Y = C/T coexistence, R = A/G coexistence.

was associated with a 2.5–408-fold increase of viral load.

When nucleotide variations were localised in relation to the secondary structure of 5′UTR, it was interesting to note that these variations were all localised in correspondence to the stem of domain II [Brown et al., 1992] (Fig. 3) and were "not compensatory" nucleotide transitions but never purine–pyrimidine transversion, thus maintaining the secondary structure typical of this region [Brown et al., 1992]. The free energy (DG) of these structures ranged between −10.6 to −15.2 vs. −10.6 of the HCV-2 prototype [Enomoto et al., 1990]. In two cases, a base insertion (A) at the same position of the loop was observed: this occurred in the only patient infected with HCV genotype 1 and in a patient infected with HCV genotype 2.

DISCUSSION

In chronic HCV infection, the relationship between HCV replication and activity of liver disease is still controversial. The induction of persistent transaminase normalisation by IFN therapy, despite ongoing HCV replication, is an infrequent event that might be related to either changes of the host immune reactivity

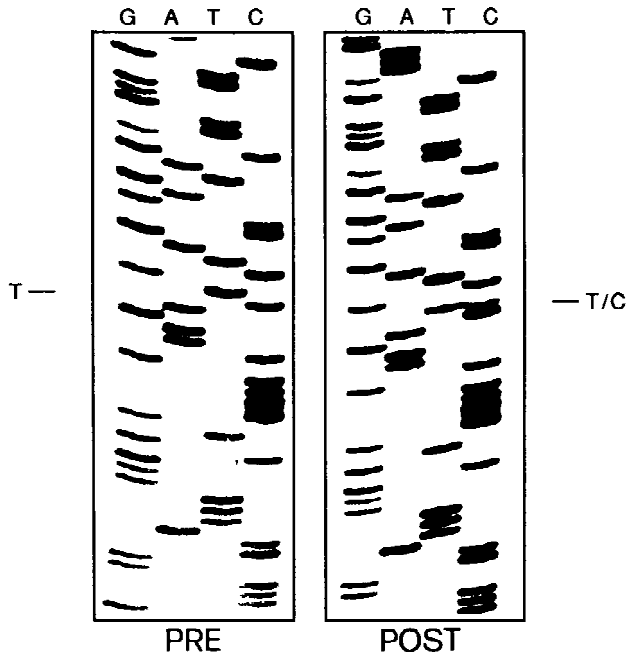

Fig. 2. Sequence comparison between sera obtained before **(pre)** and after **(post)** INF treatment in a patient showing the presence of a mixed viral population (T/C nucleotides migrating at the same electrophoretic position) detectable after therapy.

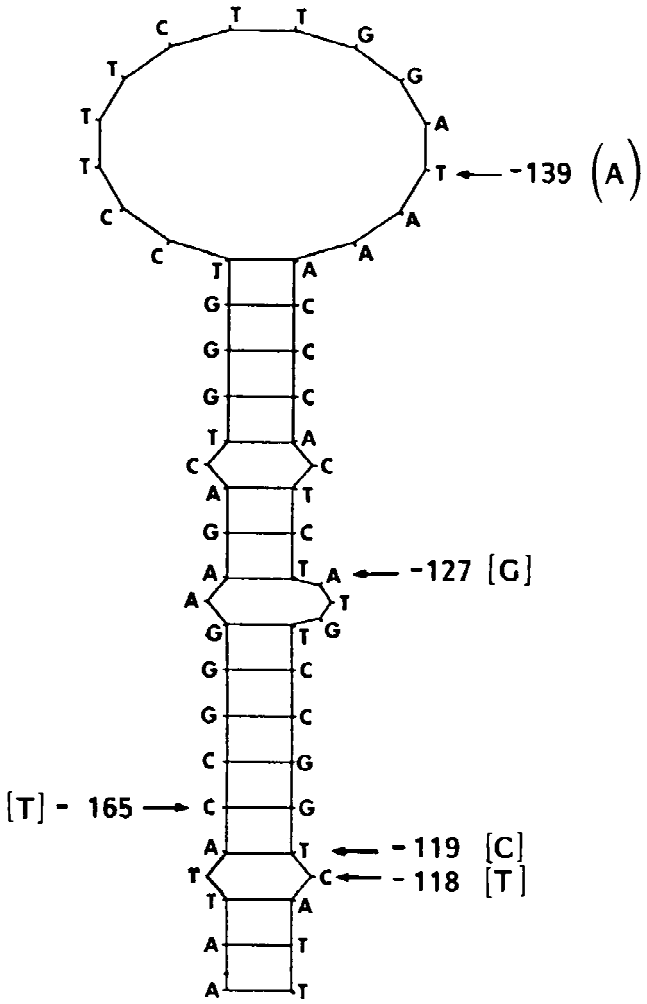

Fig. 3. Positions of nucleotide changes observed in the 5′UTR in relation to the secondary structure of this region. Parentheses refers to position of nucleotide insertion and brackets to the position of the nucleotide transition. For nucleotide numbering, see Choo et al. [1991].

or modification of viral characteristics, including significant decrease of viral load or selection of viral mutants. To verify the latter hypothesis, HCV-RNA levels before and after therapy were evaluated in parallel in a large series of patients treated with IFN who showed persistent biochemical normalisation and detectable HCV-RNA up to a year after treatment withdrawal. The possibility that traces of virus could be responsible for the observed HCV-RNA positivity by PCR was excluded because unchanged or increased HCV RNA values were found during the follow-up period.

Interestingly, all but one of the 27 patients with IFN-induced transaminase normalisation and ongoing HCV replication were infected by genotype 2, as observed in previous reports [Silini et al., 1994; Pontisso et al., 1995a; Prati et al., 1996] in the majority of untreated viraemic patients with normal ALT values. This finding suggests that particular characteristics might be possessed by this viral genotype.

The cessation of biochemical activity in our series of patients may also be due to the emergence of mutated viral strains. To explore this hypothesis, we compared viral sequences at the 5′UTR level, where the IRES activity of the virus has been localised [Wang et al., 1994]. Experimental data indicate that different HCV genotypes, showing nucleotide changes in this region, and naturally occurring mutants have a different efficiency of protein translation [Tsukiyama-Kohara et al., 1992; Feller et al., 1996; Gerotto et al., 1997]. Nucleotide variations might therefore influence the expression of the viral polyprotein and modify viral pathogenicity. The results obtained do not support this hypothesis, however, do not exclude the implication of other genomic regions of the virus that should be analysed to draw final conclusions.

An unexpected finding was the occurrence of single base transitions, localised in "hot spots" of the stem part of the 5′UTR structure, not affecting its computer-predicted stability. These observations, previously described in patients infected with genotype 2 and with LKM-1 positivity [Gerotto et al., 1994], further support the biological relevance of this region and the peculiar behaviour of genotype 2.

Heterogeneous viral strains, having single base differences at the 5′UTR level, were detected in some sera. Similar findings were reported by temperature gradient gel electrophoresis and confirmed by sequencing of several clones in sera of two of three HCV-positive patients [Lu et al., 1995]. The mixed viral population detected at the 5′UTR level in the present study seems to be correlated with higher levels of viraemia, thus allowing identification of the predomi-

nant strains by the direct sequencing approach used, without specific clinical significance.

In conclusion, comparison of virus characteristics in the same patient at the time of active liver disease and at the time of IFN-induced biochemical remission excludes that this phenomenon was induced by trivial amounts of remaining virus. Interestingly, all but one patient were infected by genotype 2 and showed several nucleotide variations in the 5′UTR. Although a characteristic sequence was not identified as far as the 5′UTR is concerned, other potentially important regions should be explored further.

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REFERENCES

Brown EA, Zhang H, Ping LH, Lemon SM (1992): Secondary structure of the 5′nontranslated region of hepatitis C virus and pestivirus genomic RNA. Nucleic Acids Research 20:5041–5045.

Casarin C, Chemello L, Bonetti P, Benvegnù L, De Moliner L, Pontisso P, Tremolada F, Alberti A, and the TriVeneto Viral Hepatitis Group (1994): Comparison of different types and doses of alpha-2a interferon for treatment of chronic hepatitis C. Journal of Hepatology Suppl 1 21:S62.

Chemello L, Bonetti P, Cavalletto L, Talato F, Donadon V, Casarin P, Belussi F, Frezza M, Noventa F, Pontisso P, Benvegnù L, Casarin C, Alberti A, and the TriVeneto Viral Hepatitis Group (1995): Randomized trial comparing three different regimens of alpha-2a interferon in chronic hepatitis C. Hepatology 22:700–706.

Chemello L, Cavalletto L, Casarin C, Bonetti P, Bernardinello E, Pontisso P, Donada C, Belussi F, Martinelli S, Alberti A (1996): Persistent hepatitis C viraemia predicts late relapse after sustained response to alpha-interferon in chronic hepatitis C. Annals of Internal Medicine 124:1058–1060.

Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby A, Barr PJ, Weiner A, Bradley DW, Kuo G, Houghton M (1991): Genetic organisation and diversity of the hepatitis C virus. Proceedings of the National Academy of Sciences of the USA 88:2451–2455.

Conjeevaram HS, Everhart JE, Hoofnagle JH (1995): Predictors of a sustained beneficial response to interferon alfa therapy in chronic hepatitis C. Hepatology 22:1326–1329.

Enomoto N, Takada A, Nakao T, Date T (1990): There are two major types of hepatitis C virus in Japan. Biochemical Biophysical Research Communications 170:1021–1025.

Feller JA, Grace K, Clarke BE, Lau JYN (1996): Variations in the translational efficiency of naturally occurring hepatitis C virus (HCV) variants. Implications for the design of antiviral therapy [abstract]. Hepatology 24:263A.

Gerotto M, Pontisso P, Giostra F, Francesconi R, Muratori L, Ballardini G, Lenzi M, Tisminetzky S, Bianchi FB, Baralle F, Alberti A (1994): Analysis of hepatitis C virus genome in patients with anti-LKM-1 autoantibodies. Journal of Hepatology 21:273–276.

Gerotto M, Pontisso P, Tisminetszky S, Alberti A, Baralle F (1997): Translational activity of the 5′UTR of HCV: Differences between genotypes, naturally occurring variants and artificial mutants [abstract]. Journal of Hepatology Suppl 1 27:73.

Lau JYN, Mizokami M, Ohno T, Diamond D, Kniffen J, Davis GL (1993): Discrepancy between biochemical and virologic responses to interferon-α in chronic hepatitis C. Lancet 342:1208–1209.

Lu M, Funsch B, Wiese M, Roggendorf M (1995): Analysis of hepatitis C virus quasispecies populations by temperature gradient gel electrophoresis. Journal of General Virology 76:881–887.

McGuiness PH, Bishop GA, Painter DM, Chan R, McCaughan W (1996): Intrahepatic hepatitis C RNA levels do not correlate with degree of liver injury in patients with chronic hepatitis C. Hepatology 23:676–687.

Pontisso P, Ruvoletto MG, Gerotto M, De Moliner S, Tisminetzky S, Chemello L, Casarin C, Baralle F, Alberti A (1995a): Genomic characterization of HCV in viremic patients with normal ALT [abstract]. Hepatology 22:343A.

Pontisso P, Ruvoletto MG, Nicoletti M, Tisminetzky S, Gerotto M, Levrero M, Artini M, Baldi M, Ballardini G, Barbara L, Bonetti P, Brillanti S, Casarin C, Chemello L, Costanzo A, De Mitri MS, Donada C, Felaco MF, Frezza M, Gaeta GB, Giostra F, Iervese T, Lo Iacono O, Milella M, Monno L, Negro F, Piccinino F, Russo G, Vaccaro A, Baralle F, Alberti A (1995b): Distribution of three major hepatitis C virus genotypes in Italy: A multicentre study of 495 patients with chronic hepatitis C. Journal of Viral Hepatitis 2:33–38.

Prati D, Capelli C, Zanella A, Mozzi F, Bosoni P, Pappalettera M, Zanuso F (1996): Influence of different hepatitis C virus genotypes on the course of asymptomatic hepatitis C virus infection. Gastroenterology 110:178–183.

Silini E, Bono F, Cividini A, Cerino A, Sanno B, Rossi S, Belloni S, Brugnetti B, Civardi E, Salvaneschi L, Mondelli M (1994): Differential distribution of hepatitis C virus genotypes in patients with and without liver functional abnormalities. Hepatology 21:285–290.

Simmonds P (1995): Variability of hepatitis C virus. Hepatology 21: 570–583.

Tisminetzky S, Gerotto M, Pontisso P, Chemello L, Prescott LE, Rose KA, Baralle F, Simmonds P, Alberti A (1995): Comparison of genotyping and serotyping methods for the identification of hepatitis C virus types. Journal of Virological Methods 55:303–307.

Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A (1992): Internal ribosome entry site within hepatitis C virus RNA. Journal of Virology 66:1476–1483.

Wang C, Sarnow P, Siddiqui A (1993): Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. Journal of Virology 67:3338–3344.

Wang C, Sarnow P, Siddiqui A (1994): A conserved helical element is essential for internal initiation of translation of hepatitis C virus RNA. Journal of Virology 68:7301–7307.